

Association of *STAT4* polymorphisms with hepatitis B virus infection and clearance in Chinese Han population

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Abstract Genome-wide association study indicates that *STAT4* is a plausible candidate for an association study with HBV-related liver diseases. We aimed to examine the roles of *STAT4* polymorphisms on HBV-related liver diseases in a Chinese Han population. We selected 13 SNPs in *STAT4* based on the HapMap database to investigate their associations in 3,033 participants. SNP rs7574865 was significantly associated with HBV infection [odds ratio (OR) 1.15; 95 % confidence interval (CI) 1.00, 1.31; $P = 0.046$] and clearance (OR 1.17; 95 % CI 1.02, 1.33; $P = 0.028$). Further, haplotype-based association analysis indicated that the haplotype CTCTT, formed by SNPs rs8179673, rs7574865, rs4274624, rs11889341, and rs10168266, was significantly associated with HBV infection (OR 0.87; 95 % CI 0.76, 0.99; $P = 0.022$) and clearance (OR 0.86; 95 % CI 0.75, 0.99; $P = 0.018$). Bioinformatics analysis of these SNPs predicted that they participate in transcriptional

regulation. Taken together, our findings demonstrate that variants in *STAT4* play a critical role in HBV infection and clearance in the Chinese Han population.

Keywords *STAT4* · Chronic hepatitis B virus · SNPs · Infection · Clearance

Abbreviations

HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
CHB	Chronic hepatitis B
LC	Liver cirrhosis
STAT4	Signal transducer and activator of transcription 4
HBsAg	Hepatitis B surface antigen
HBsAb	Hepatitis B surface antibody
HBeAg	Hepatitis B e antigen
HBeAb	Hepatitis B e antibody
HBcAb	Hepatitis B core antibody
HCsAb	Hepatitis C surface antibody
ALT	Serum alanine aminotransferase
AsC	Asymptomatic carriers

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Introduction

Infection with HBV is a major public health issue throughout the world, especially in Asian countries (Schweitzer et al. 2015). Despite the effective use of the potent HBV vaccine and antiviral treatments, HBV infection maintains a high prevalence worldwide, with an estimated 240 million people affected chronically in 2015 (World Health Organization 2015; <http://www.who.int/mediacentre/factsheets/fs204/en/>), and is the world's tenth leading cause of death (Trepo et al. 2014). Approximately 60 % of hepatocellular carcinoma (HCC) is associated with chronic hepatitis B

Table 1 Clinical demographics of subjects in each subgroup

Characteristic	Healthy	Clearance	AsC	CHB	LC	HCC
Sample size	749	674	388	853	296	73
Age (years)	37.7 ± 12.4	39.3 ± 11.3	36.7 ± 11.3	39.1 ± 11.4	47.8 ± 9.8	51.8 ± 11.4
Male (%)	450 (60.1)	439 (65.1)	254 (65.5)	646 (75.7)	236 (79.7)	61 (83.6)
HBV-DNA ($\times 10^8$ copies/mL)	–	–	3.5 ± 11.5	2.0 ± 8.5	0.5 ± 3.6	0.1 ± 0.5
HBsAg ($\times 10^3$ IU/mL)	–	–	3.3 ± 3.9	2.8 ± 3.4	1.0 ± 1.6	0.6 ± 1.3
HBsAb (mIU/mL)	–	299.6 ± 412.2	1.77 ± 8.9	2.1 ± 12.4	3.5 ± 25.5	5.5 ± 23.3
HBcAb (S/CO)	–	7.2 ± 6.0	17.6 ± 28.2	32.5 ± 312.8	36.3 ± 47.4	64.6 ± 55.1
HBeAg (PEIU/mL)	–	–	60.3 ± 114.3	55.5 ± 354.5	25.2 ± 255.7	10.4 ± 37.0
HBeAb (S/CO)	–	1.1 ± 5.5	11.2 ± 24.9	7.7 ± 14.5	7.3 ± 72.8	7.3 ± 17.8
ALB (g/L)	47.9 ± 6.6	47.8 ± 6.7	48.1 ± 6.8	53.5 ± 177.4	56.3 ± 248.0	38.0 ± 6.4
ALT (U/L)	24.7 ± 27.0	25.5 ± 25.8	57.7 ± 135.0	73.7 ± 125.9	53.2 ± 127.4	60.0 ± 68.1
TBIL (μ mol/L)	13.3 ± 21.6	12.9 ± 8.5	18.9 ± 45.6	31.8 ± 87.7	35.0 ± 58.3	163.4 ± 1077.1
DBIL (μ mol/L)	5.2 ± 22.3	6.1 ± 26.2	8.1 ± 29.3	14.7 ± 43.4	18.5 ± 36.1	20.5 ± 54.3
IBIL (μ mol/L)	9.4 ± 17.4	10.7 ± 20.2	10.7 ± 14.9	14.8 ± 25.3	16.5 ± 22.9	17.2 ± 30.4

Continuous variables are shown as mean ± standard deviation

ALB albumin, ALT alanine aminotransferase, AsC asymptomatic carrier, CHB chronic hepatitis B, DBIL direct bilirubin, HCC hepatocellular carcinoma, IBIL indirect bilirubin, LC liver cirrhosis, TBIL total bilirubin

(CHB), and the infection is responsible for many cases of liver cirrhosis (LC) (Lai et al. 2003; Perz et al. 2006).

Infection with HBV and its progression to LC and HCC are influenced by viral, environmental, and host factors (Thursz 2001). Genome-wide association studies (GWAS) have been recently used increasingly to identify genetic variants in HBV-related liver diseases, which revealed several novel common variants located in ubiquitin-conjugating enzyme E2L3 (Hu et al. 2013), solute carrier family 10 member 1 (Peng et al. 2015), glutamate receptor ionotropic kainate 1 (Li et al. 2012), kinesin family member 1B (Li et al. 2012), and signal transducer and activator of transcription 4 (*STAT4*) (Jiang et al. 2013). However, many of these newly identified susceptibility gene findings have not been replicated in independent studies.

STAT4 is located on chromosome 2q32.3 and encodes a crucial transcription factor (Zhong et al. 1994). Several cytokines, such as interleukin (IL)-2 (Wang et al. 1999), IL-12 (Bacon et al. 1995) and interferon (IFN)- α/β (Nguyen et al. 2002), are reported to activate *STAT4* for transmitting signals to its downstream target genes. In natural killer (NK) and T cells, *STAT4* is phosphorylated predominately by the binding of IL-12 and its receptor. Then the phosphorylated *STAT4* stimulates NK and T cells to produce IFN- γ , an important cytokine for antiviral replication and antitumor activity (Wang et al. 2015). Moreover, it has been reported that *STAT4* plays critical roles in other viral infections, such as respiratory syncytial virus (Dulek et al. 2014), herpes simplex virus (HSV) 2 (Svensson et al. 2012), and lymphocytic choriomeningitis virus (Holz

et al. 1999). A recent GWAS found that SNP rs7574865 in *STAT4* was associated with HBV infection and HBV-related HCC (Jiang et al. 2013), but this association has not been replicated yet in any other Chinese Han population.

To further investigate the association of *STAT4* polymorphisms with HBV-related liver diseases, we performed an association study of single and multiple SNPs within *STAT4* in a large Chinese Han sample.

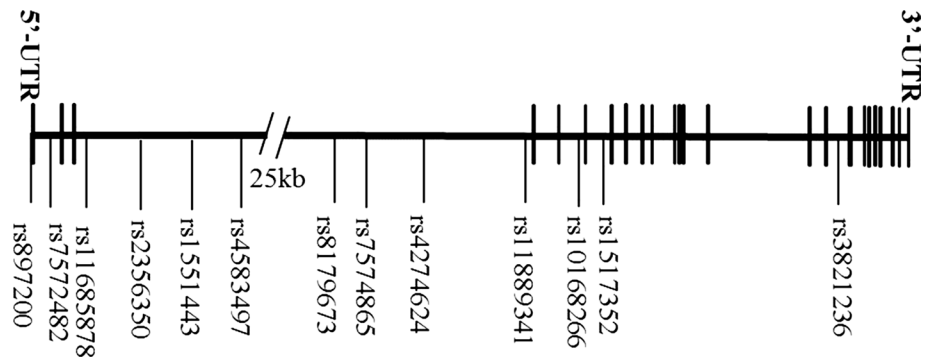
Materials and methods

Subject recruitment and demographic characteristics

This case–control study included 3033 Chinese Han individuals comprising 1610 with chronic HBV infection (CHBVI) and 1423 uninfected control subjects (Table 1). A detailed description of this sample has been reported previously (Tao et al. 2015).

Based on the laboratory testing results and the “Guideline of Prevention and Treatment for Chronic Hepatitis B” defined by the Chinese Medical Association (Chinese Society of and Chinese Society of Infectious Diseases 2011), 1610 CHBVI patients could be identified as asymptomatic carriers (AsC; $n = 388$), CHB ($n = 853$), LC ($n = 296$), or HCC ($n = 73$). The diagnostic criteria for these HBV-related liver diseases were as follows: (1) AsC: seropositive for either HBsAg or HBV-DNA and persistently normal ALT; (2) CHB: seropositive for either HBsAg or HBV-DNA and recurrently abnormal or continuously elevated ALT for >6 months; (3) LC: presence of ascites,

Fig. 1 Location of selective SNPs in *STAT4* with a total length of 122 kb. Exons or UTRs for *STAT4* are shown as black bars and introns as horizontal black lines. This figure is shown in its original scale



hypersplenism, elevated ALT, and appropriate imaging findings; (4) HCC: abnormal serum alpha-fetoprotein and imaging findings. The 1423 control subjects were classified as healthy ($n = 749$) or HBV clearance ($n = 674$). The definition of healthy subjects and clearance was: (1) healthy: negative for HBsAg, HBcAb, and HBsAb; and (2) clearance: negative for HBsAg but positive for HBcAb and HBsAb.

Subjects were excluded if they were: (1) co-infected with any other hepatitis virus; (2) suffered from any other type of cancer or autoimmune disease; (3) positive for human immunodeficiency virus; (4) effectively immunized against HBV; (5) not Chinese Han ethnicity; or (6) pregnant.

DNA extraction, SNP selection and genotyping, and quality control

Genomic DNA was extracted from 3 ml of peripheral blood of each subject using the Qiagen DNA purification kit. Selection of 13 SNPs in *STAT4* was based on the HapMap database (Release #27) with a minor allele frequency (MAF) >0.1 in the Chinese population (Fig. 1; Table S1). Of the 13 selected variants, SNPs rs3821236, rs1517352, rs10168266, rs11889341, rs4274624, rs7574865, rs8179673, rs11685878, rs7572482, and rs897200 have been reported to be associated with autoimmune or viral disease, including systemic lupus erythematosus (SLE) (Kawasaki et al. 2008), inflammatory bowel disease (Jostins et al. 2012), primary biliary cirrhosis (PBC) (Joshita et al. 2014), autoimmune Addison's disease (Mitchell et al. 2014), Behcet's disease (Hou et al. 2012), HBV-related HCC (Jiang et al. 2013), or HSV-2 infection (Svensson et al. 2012).

All SNPs included in the study were genotyped in a 384-well microplate format using the *TaqMan* SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA). The genotyping rate for each SNP was required to be $>95\%$, and the P value for the Hardy–Weinberg equilibrium was set to >0.05 .

Literature search for meta-analyses on the association of rs7574865 with HBV infection and clearance

Our meta-analysis was performed according to the criteria suggested in the PRISMA guidelines (Moher et al. 2009). We searched PubMed and Web of Science for all eligible studies that could be included in this study. The key words used in the search strategy were: “rs7574865” or “*STAT4*” or “signal transducer and activator of transcription 4” and “hepatitis B” or “hepatitis B virus” or “HBV”. The identified reports were checked individually for reference to studies that were not found in the abovementioned electronic databases. When different samples of the same population were included in a study, each one was treated as an independent one in this report. For each study, the following data were extracted: author, publication year, ethnicity, study design, genotyping method, sample size, mean age, and sex ratio.

The inclusion criteria for the meta-analysis were that: (1) the study was designed as a case–control study; (2) the study provided the number of CHBVI patients, healthy individuals, and subjects with HBV clearance, and the allele frequency of rs7574865; (3) the study presented sufficient information for calculating the odds ratio (OR) and the 95 % confidence interval (CI).

Statistical analysis

The population admixture of subjects was assessed by principal component (PC) analysis using 291 ancestry informative markers (AIMs) selected from the Illumina arrays. For individual SNP-based association analysis, the association of *STAT4* polymorphisms with HBV-related liver diseases was performed under an additive genetic model using PLINK (v. 1.07) (Purcell et al. 2007). The ORs and 95 % CIs were calculated by logistic regression with age, sex, and the first seven PCs as covariates. After Bonferroni correction for multiple tests, the appropriate significance of individual SNP was set to $P = 3.8 \times 10^{-3}$.

Table 2 Association of SNPs in *STAT4* with HBV infection and clearance under additive genetic model

SNP ID	Effect allele	HBV Infection				HBV Clearance			
		EAF		OR (95 %CI)	P value	EAF		OR (95 %CI)	P value
		CHBVI	Healthy			CHBVI	Clearance		
rs3821236	G	0.57	0.56	1.04 (0.92, 1.18)	0.514	0.57	0.54	1.13 (0.99, 1.28)	0.065
rs1517352	C	0.54	0.52	1.06 (0.93, 1.20)	0.397	0.54	0.55	0.94 (0.82, 1.07)	0.336
rs10168266	C	0.70	0.66	1.19 (1.04, 1.36)	0.013	0.70	0.67	1.15 (1.00, 1.31)	0.043
rs11889341	C	0.70	0.67	1.18 (1.03, 1.34)	0.018	0.70	0.67	1.15 (1.01, 1.32)	0.041
rs4274624	T	0.69	0.66	1.13 (0.99, 1.29)	0.056	0.69	0.66	1.15 (1.01, 1.32)	0.050
rs7574865	G	0.70	0.67	1.15 (1.00, 1.31)	0.046	0.70	0.66	1.17 (1.02, 1.33)	0.028
rs8179673	T	0.69	0.67	1.14 (0.99, 1.30)	0.047	0.69	0.66	1.15 (1.01, 1.32)	0.040
rs4583497	G	0.69	0.70	0.91 (0.79, 1.04)	0.157	0.69	0.68	1.02 (0.89, 1.17)	0.798
rs1551442	A	0.72	0.73	0.96 (0.83, 1.10)	0.542	0.72	0.71	1.07 (0.93, 1.23)	0.361
rs2356350	G	0.51	0.50	1.05 (0.93, 1.19)	0.430	0.51	0.51	1.12 (0.99, 1.27)	0.081
rs11685878	C	0.53	0.51	1.06 (0.94, 1.21)	0.337	0.53	0.51	1.06 (0.93, 1.21)	0.397
rs7572482	A	0.54	0.56	0.90 (0.80, 1.03)	0.121	0.54	0.56	0.91 (0.80, 1.04)	0.155
rs897200	A	0.54	0.56	0.91 (0.81, 1.04)	0.164	0.54	0.56	0.93 (0.81, 1.06)	0.256

OR and *P* value were adjusted for age, sex, and first seven principal components

Significant differences are shown in boldface type

AsC asymptomatic carrier, CHB chronic hepatitis B, CHBVI chronic hepatitis B virus infection (includes AsC, CHB, LC, and HCC), CI confidence interval, EAF effect allele frequency, HCC hepatocellular carcinoma, LC liver cirrhosis, OR odds ratio

(0.05/13). Pairwise linkage disequilibrium (LD) was determined by the D' value, and haplotype blocks were generated by the Haploview software (v. 4.2) (Barrett et al. 2005). The association of each haplotype with HBV-related liver diseases was analyzed using R package “haplo.stats” (<http://cran.r-project.org/web/packages/haplo.stats>; Yang and Li 2014). The *P* value was given by the score test under the same genetic model and covariates as used in the individual SNP-based association analysis (Schaid et al. 2002). The significance of each haplotype was adjusted by the number of major haplotypes (frequency >5 %) included in the analysis. For the SNPs within the significant haplotypes, variant effects on the regulation of transcription were analyzed using HaploReg (v. 2) (http://www.broadinstitute.org/mammals/haploreg/haploreg_v2.php). In this database, evolutionarily conserved regions are predicted by genome evolutionary rate profiling (GERP) (Ward and Kellis 2012), the enhancer histone mark prediction was based on the evidence of local H3K4Me1 modification, and variant effects on regulatory motifs were evaluated according to TRANSFAC, JASPAR and protein-binding microarray experiments (Ward and Kellis 2012).

Meta-analysis was performed to assess the association of rs7574865 with HBV infection and clearance using Comprehensive Meta-Analysis software (v. 2.0; Biostat Inc., Englewood, NJ, USA, as we described previously (Ma et al. 2015a, b)). Heterogeneity among the studies was assessed by Cochran's Q and I^2 statistic tests (Higgins et al. 2003). If the

P value of the Q test is <0.1, indicating heterogeneity among the studies, the random-effects model is more applicable. Otherwise, use of the fixed-effects model is encouraged. The I^2 test is used to analyze the effect of heterogeneity and the degree of inconsistency among the studies. If Cochran's Q *P* value is >0.1 and the I^2 value is ≤ 50 %, we consider that there exists no strong evidence for heterogeneity (Higgins et al. 2003; Taylor et al. 2015). In this work, both the random-effects and fixed-effects models were used in the meta-analysis. The Z statistical test was used to calculate the significance of pooled ORs. A funnel plot was applied to assess potential publication bias. Sensitivity analysis was performed to detect the stability of results. The significance level was set to $P < 0.05$ (two-sided) for meta-analysis.

Results

Association analysis of *STAT4* polymorphisms with HBV infection and clearance

To identify the association of *STAT4* polymorphisms with HBV infection and clearance, we compared individual SNP-based association analysis of 13 SNPs in 1610 CHBVI patients vs. 749 healthy subjects and 1610 CHBVI patients vs. 674 clearance subjects under an additive genetic model after adjusting for age, sex, and the first seven PCs (Table 2).

Table 3 Haplotype analysis of *STAT4* polymorphisms with HBV infection and clearance

No	Haplotype	Frequency (CHBVI)	Frequency (Healthy)	Frequency (Clearance)	<i>P</i> * (CHBVI vs. Healthy)	Hap Score	<i>P</i> * (CHBVI vs. Clearance)	Hap Score
1	CTCTT	0.280	0.310	0.314	0.022	-2.290	0.018	-2.370
2	TGTCC	0.667	0.638	0.645	0.072	1.796	0.181	1.337

SNP order in the haplotype: rs8179673-rs7574865-rs4274624-rs11889341-rs10168266

Hap Scores and *P* values were adjusted for age, sex, and the first seven principal components under the additive genetic model

*CHBVI vs. Healthy or Clearance; significant *P* values after Bonferroni correction for two major haplotypes (frequency >0.05) are 0.025 (given in boldface type)

We found that SNP rs7574865 was significantly associated with HBV infection risk (OR 1.15; 95 % CI 1.00, 1.31; *P* = 0.046), providing an independent replication of a previous report (Jiang et al. 2013). Furthermore, this SNP was negatively correlated with HBV clearance (OR 1.17; 95 % CI 1.02, 1.33; *P* = 0.028). That is, rs7574865 plays a role in HBV susceptibility, as well as a retarding role in HBV clearance.

An additional four SNPs showed a nominally significant correlation with HBV infection and clearance (Table 2). When comparing CHBVI with the healthy group, we obtained the OR and its corresponding *P* value for SNPs rs10168266 (OR 1.19; *P* = 0.013), rs11889341 (OR 1.18; *P* = 0.018), rs4274624 (OR 1.13; *P* = 0.056), and rs8179673 (OR 1.14; *P* = 0.047). As for the HBV clearance, the ORs and their corresponding *P* values were as follows: rs10168266 OR 1.15; *P* = 0.043; rs11889341 OR 1.15; *P* = 0.041; rs4274624 OR 1.15; *P* = 0.050; and rs8179673 OR 1.15; *P* = 0.040. Thus, all of the SNPs showed a risk effect on HBV infection and clearance.

Association analysis of *STAT4* polymorphisms with disease progression

To reveal the relation of *STAT4* variants to the progression of HBV-related liver diseases, we performed the following three comparisons: (1) AsC vs. CHB; (2) CHB vs. LC; and (3) LC vs. HCC. We found no significant association of the *STAT4* variants with disease progression after adjusting for age, sex, and the first seven PCs (detailed results not shown).

Haplotype analysis of *STAT4* polymorphisms with HBV infection and clearance

Haplotype-based association analysis was performed for all SNPs in *STAT4* regardless of whether they were significant or non-significant according to the individual association analysis. One haplotype, CTCTT, formed by rs8179673, rs7574865, rs4274624, rs11889341, and rs10168266, showed a significant association with HBV

infection and clearance (Table 3; Fig. 2). When comparing the CHBVI with the healthy group, the haplotype CTCTT was significantly associated with HBV infection (Hap Score = -2.290; *P* = 0.022). Similarly, the haplotype CTCTT was significantly associated with HBV clearance when comparing the CHBVI with the clearance group (Hap Score = -2.370; *P* = 0.018). Together, these results indicate that the haplotype formed by the minor alleles of the abovementioned SNPs is protective against HBV susceptibility and beneficial for HBV clearance.

Meta-analysis of association of rs7574865 with HBV infection and clearance

To determine the effects of rs7574865 on HBV infection and clearance with a large sample, we performed meta-analysis for those reported studies along with the current one. Based on this search strategy, we included 10 reported studies and one unpublished data set totaling 8944 CHBVI patients, 8451 healthy individuals, and 2081 subjects with HBV clearance (Table S2). Under the fixed-effects model, rs7574865 was significantly associated with HBV infection (pooled OR 1.14; 95 % CI 1.07, 1.21; pooled *P* = 3.8×10^{-5} ; Fig. 3a) and clearance (OR 1.20; 95 %CI 1.07, 1.35; *P* = 0.002; Fig. 4a). Similarly, we performed a meta-analysis under the random-effects model, which again revealed rs7574865 to be significantly associated with HBV infection (pooled OR 1.14; 95 % CI 1.07, 1.21; pooled *P* = 3.8×10^{-5} ; Fig. 3b) and clearance (pooled OR 1.25; 95 % CI 1.01, 1.55; pooled *P* = 0.044; Fig. 4b). Considering that we found no significant evidence for the presence of heterogeneity among the studies for the meta-analysis of HBV infection (*P* = 0.82; *I*² = 0) and clearance (*P* = 0.19; *I*² = 34 %), we believed the results from either model are acceptable, and both models provide further evidence for the significant association of rs7572865 with HBV infection and clearance. Next, we performed sensitivity analysis by sequentially omitting individual studies under the fixed-effects model and found that the *P* value remained significant for all cases (data not shown).

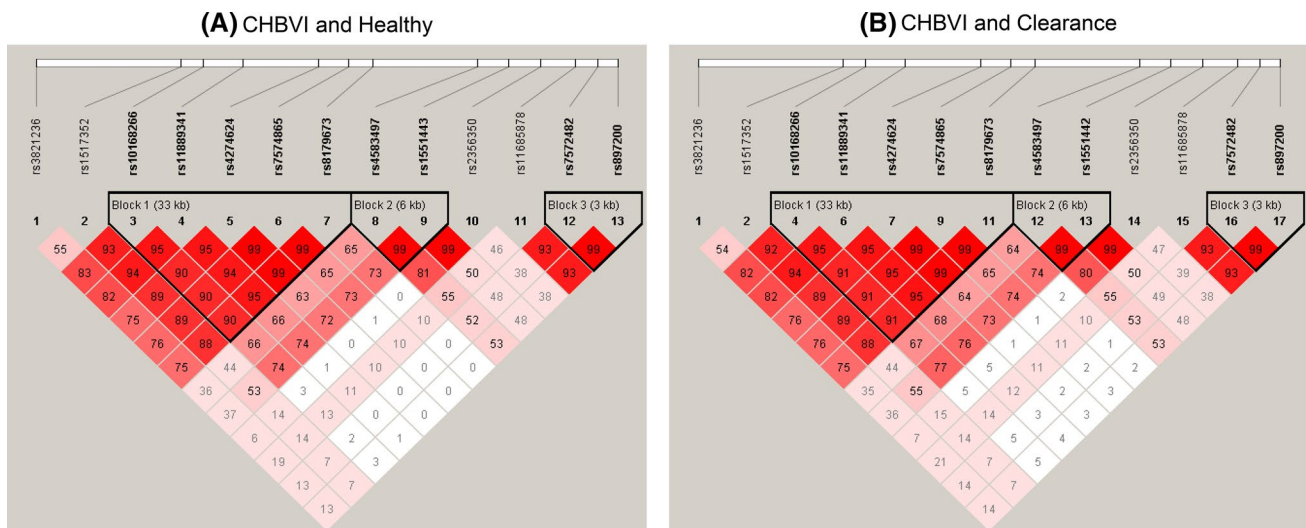


Fig. 2 LD maps and haplotype blocks of *STAT4* generated by Haploview software (v. 4.2). The LD map was determined by the D' value ($D' > 0.9$), which was drawn from the genotypic data of the CHBVI and healthy subjects (a) and the genotypic data of the CHBVI and

clearance subjects (b). LD linkage disequilibrium, CHBVI chronic HBV infection (includes AsC, CHB, LC, and HCC), AsC asymptomatic carrier, CHB chronic hepatitis B, LC liver cirrhosis, HCC hepatocellular carcinoma

Function prediction analysis of SNPs in *STAT4*

We applied the HaploReg tool (Ward and Kellis 2012) to predict the function of the five SNPs in the significant haplotype CTCTT (Table S3). Of the five, SNP rs7574865 was predicted to be conserved based on the GERP; SNP rs11889341 was associated with histone modifications in B cells from peripheral blood that were suggestive of enhancer activity. In addition, four SNPs (rs10168266, rs11889341, rs7574865, and rs8179673) were predicted to affect specific regulatory motifs such as Foxp1, Foxo3, HNF1, and HNF4. These predictions indicated that the SNPs have important effects on transcription by influencing the binding of transcription factors or regulatory motifs.

Discussion

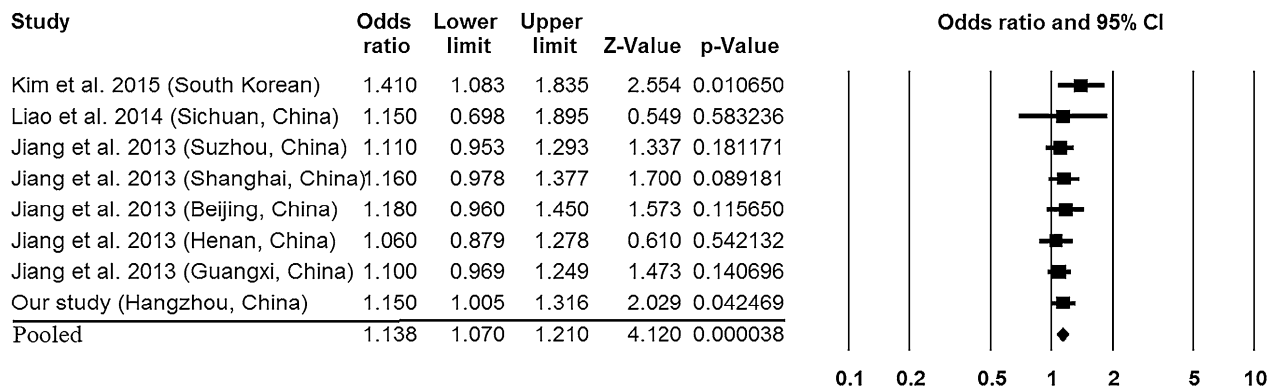
A recent GWAS study identified *STAT4* as a susceptibility gene for HBV infection and HBV-related HCC in the Chinese Han population (Jiang et al. 2013). However, Liao et al. failed to validate such an association using 1312 Chinese Han individuals (Liao et al. 2014). To determine whether this gene indeed plays any role in HBV-related liver diseases, we carried out this study with a much larger sample.

In individual SNP-based association analysis under the additive genetic model, SNP rs7574865 in *STAT4* not only increased the probability of HBV infection (OR = 1.15; $P = 0.046$) but also reduced the probability of viral clearance (OR = 1.17; $P = 0.028$). The meta-analysis of the

data on this SNP, incorporating all the data from 8944 CHBVI patients, 8451 healthy and 2081 HBV clearance individuals, also indicated SNP rs7574865 to be a risk factor for HBV infection and an inhibitor of clearance. During the preparation of this manuscript, Lu et al. (2015) reported that rs7574865 is related to HBV clearance with 596 subjects, whose result is consistent with ours. It has also been reported that rs7574865/G was significantly associated with lower mRNA values for *STAT4* (Jiang et al. 2013). Because *STAT4* was able to bind to the first intron of interferon-gamma, higher *STAT4* expression was expected to enhance the activation of IFN- γ , a soluble cytokine with important effects on modulating antiviral activity and immunoregulatory functions (Morinobu et al. 2002), leading to the increased ability for HBV clearance and resistance to viral infection. Also, we found SNPs rs10168266, rs11889341, rs4274624, and rs8179673 to be nominally associated with HBV infection and viral clearance. It has been reported that SNPs rs10168266, rs11889341, and rs8179673 are significantly correlated with PBC and SLE susceptibility (Joshita et al. 2014; Kawasaki et al. 2008); and SNP rs4274624 is related to susceptibility to autoimmune Addison's disease (Mitchell et al. 2014). These results suggest that *STAT4* plays dual roles in autoimmune disease and viral infection.

We also investigated the roles of *STAT4* polymorphisms in each stage of liver disease progression. None of the SNPs showed a significant association, which indicates that *STAT4* plays different roles at different stages of HBV infection. *STAT4* protects against HBV infection and increases HBV clearance, but it may play no significant role in disease progression. However, in a recent

(A)



(B)

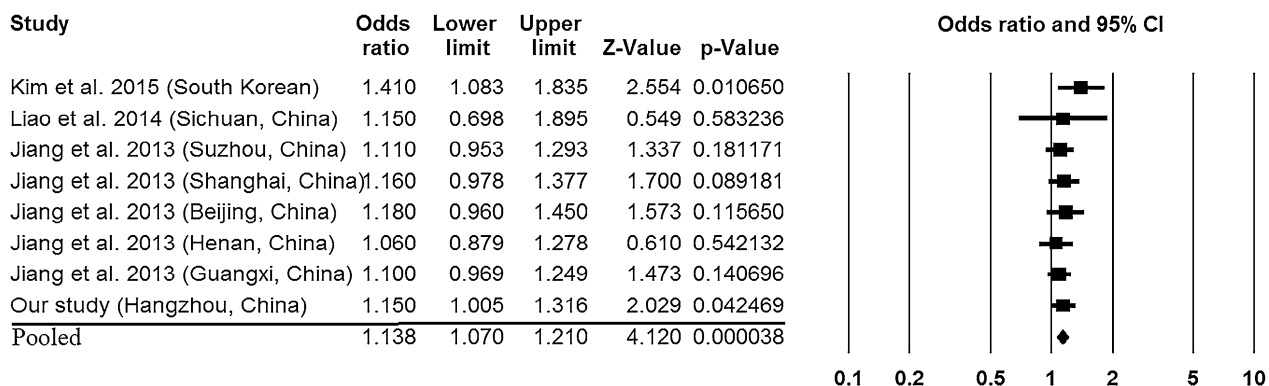


Fig. 3 Forest plots of meta-analysis results for the association of rs7574865 with HBV infection in the CHBVI and healthy groups under the fixed-effects model (a) and the random-effects model (b). The Z and P values of each study are shown by rows. The central ver-

tical solid line represents ORs that equal 1 under the null hypothesis. The ORs and 95 % CIs are represented by the square and horizontal bars, respectively. The pooled OR is indicated by the diamond

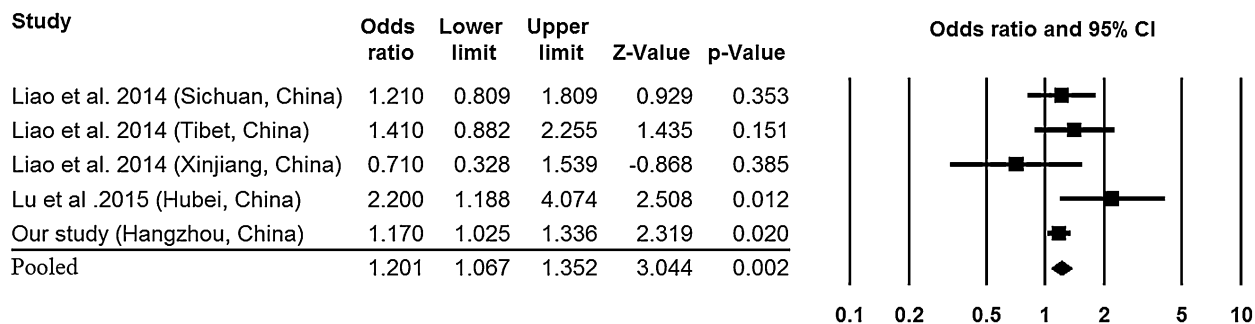
comparison of 712 LC patients and 2601 HBV-positive controls, Jiang et al. (2015) found that rs7574865 was significantly associated with disease progression, which is inconsistent with our findings. This discrepancy might be caused by a relatively small sample in a few HBV-related liver disease categories, and a large sample is required for future study.

Further, we performed haplotype-based association analysis and found a high protective CTCTT haplotype formed by rs8179673-rs7574865-rs4274624-rs11889341-rs10168266, which showed significant activity against HBV infection (Hap Score = -2.290; P = 0.022) and increased the probability of viral clearance (Hap Score = -2.370; P = 0.018). This suggested that these five SNPs jointly contribute to HBV infection and clearance, although they were not significantly associated with HBV-related liver

diseases after Bonferroni correction based on individual SNP-based association analysis.

We then evaluated the potential biological functions of these five SNPs located in noncoding regions using the HaploReg tool (Ward and Kellis 2012). It predicted that SNP rs11889341 influences the enhancer activity in B cells on the basis of its location with a histone modification. Additionally, regulatory motif prediction suggested that SNPs rs10168266, rs11889341, rs7574865, and rs8179673 affect the affinity for binding of transcription factors. Many transcription factors are associated with viral infection and liver diseases. For rs11889341, the alternate allele increases affinity for Foxo3, which regulates CD8 T cell responses during chronic viral infection (Sullivan et al. 2012). For rs7574865, the alternate allele is predicted to have a lower affinity for Foxp1, which is reported to be associated with

(A)



(B)

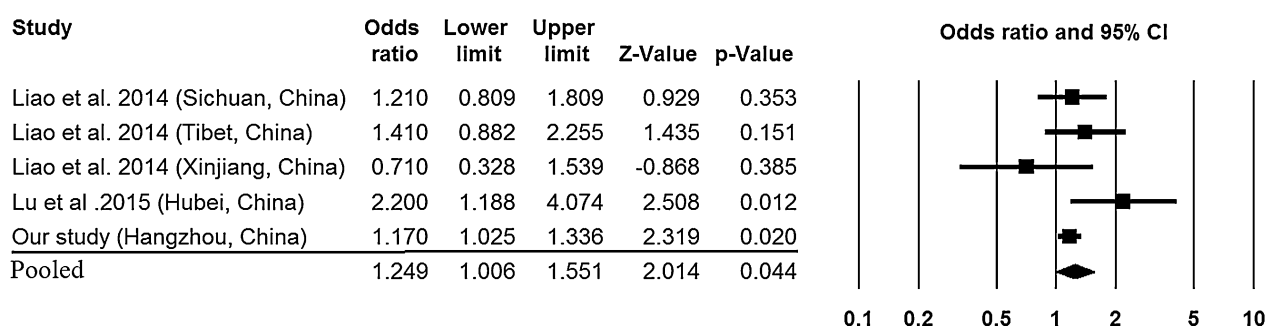


Fig. 4 Forest plots of meta-analysis results for the association of rs7574865 with HBV clearance in the CHBVI and clearance groups under the fixed-effects model (a) and the random-effects model (b). The Z and P values of each study are shown by rows. The central ver-

tical solid line represents ORs that equal 1 under the null hypothesis. The ORs and 95 % CIs are represented by the square and horizontal bars, respectively. The pooled OR is indicated by the diamond symbol

the risk of LC progression to HBV-related HCC (Li et al. 2015). We suspect that rs7574865 influences oncogenesis by changing the affinity for Foxp1. For rs8179673, its alternate allele is predicted to have lower binding of transcription factors HNF1 and HNF4 to *STAT4*. HNF1 α repression can lead to activation of JAK/STAT pathways and facilitate the progression of chronic liver diseases (Qian et al. 2015), and HNF4 also plays an important role in HBV replication (Shin et al. 2014). However, it remains to be determined how these five SNPs contribute to HBV infection and clearance by affecting *STAT4* transcriptional regulation.

In conclusion, this study confirmed the association of *STAT4* polymorphisms with HBV susceptibility and viral clearance in a much larger sample than previously studied. In addition, haplotype-based association analysis revealed a highly protective CTCTT haplotype, which is also significantly associated with HBV infection and clearance. Finally, functional prediction analysis using the HaploReg tool indicated that these five SNPs likely play critical roles

in the regulation of transcription of the *STAT4* gene. However, further functional study is required to validate these predictions.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest regarding this report.

Research involving human participants and/or animals *Statement of human rights* All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. *Statement on the welfare of animals* Not applicable.

Informed consent Informed consent was obtained from all individual participants included in the study and the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine approved the project.

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